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The Cuticle on Gametophytes of *Tmesipteris sigmatifolia*

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Abstract.—The surface of gametophytes of *Tmesipteris sigmatifolia* from axenic culture were examined with electron microscopy and histochemistry. A lipid layer, which gave a positive reaction with lipid stains, covers the surface of these gametophytes. In apical regions of the gametophyte the lipid coating, the cuticle, is less than half the thickness of the wall it covers. The wall was not stained with lipid stains, but did stain for cellulose and polyphenolic materials. A cuticle on the surface of *Tmesipteris* gametophytes indicates that a cuticle is characteristic of gametophytes in the Psilotaceae because gametophytes of both *Psilotum* and *Tmesipteris* are now known to have them.

Key Words.—*Tmesipteris*, gametophyte, cuticle

The gametophytes of the Psilotaceae are different from the typical photosynthetic fern gametophytes because they are subterranean, mycorrhizal, and long-lived. They are cylindrical gametophytes that often branch (Holloway, 1917; Lawson, 1917b). Their surface is of interest because these gametophytes grow in soil or humus for long periods of time.

Early studies reported the cuticularization of the surface of *Psilotum* gametophytes (Darnell-Smith, 1917; Holloway, 1939) and Bierhorst (1971) demonstrated lipid on their surface using Sudan IV. The structure of the surface wall of these gametophytes, including the lipid materials, was examined with histochemical techniques and electron microscopy by Whittier and Peterson (1995). It was concluded that the surface lipid layer is best considered a cuticle.

Less is known about the surface of *Tmesipteris* gametophytes. Holloway (1917) illustrated the surface wall as being thicker than the inner walls of these gametophytes and Lawson (1917a) commented on and illustrated this thicker wall. Neither study reported the cuticularization of the gametophyte surface. Because a lipid layer was not demonstrated in these studies, the present study was initiated to determine if lipids are associated with the surface of *Tmesipteris* gametophytes.

Materials and Methods

Gametophytes of *Tmesipteris sigmatifolia* Chinnock were grown on 25 ml of nutrient medium in 25 × 150 mm culture tubes with screw caps, which were tightened to reduce moisture loss. The nutrient medium contained 100 mg NH_4Cl, 100 mg $MgSO_4 \cdot 7H_2O$, 40 mg $CaCl_2$, and 100 mg K_2HPO_4 per liter. In addition, a liter of medium contained 4 ml of a FeEDTA solution (Sheat *et al.*, 1959), 0.5 ml of a minor element solution (Whittier and Steeves, 1960), and 5 g of glucose. The medium was solidified with 1.1% agar and was at pH 6.0 before autoclaving. The gametophytes were cultured in the dark at 23 ± 1°C until used.

For transmission electron microscopy (TEM) the gametophyte tissue was fixed overnight on ice in a 1:1 solution of 4% glutaraldehyde and 10% acrolein in 0.07M phosphate buffer (pH 6.8). The tissue was postfixed in 1% osmium tetroxide in 0.07M phosphate buffer (pH 6.8) on ice for 2 hr, followed by dehydration in a graded acetone series and embedded in Spurr's resin (Spurr, 1969). Thin sections were picked up on stainless steel grids and stained with a saturated solution of uranyl acetate in methanol followed by lead citrate (Venable and Coggeshill, 1965).

A variety of methods were used to prepare the gametophyte material for light microscopy. Hand sections or pieces of living gametophytes were employed for some histochemical tests. Thick sections (0.5 μm) of gametophytes in Spurr's resin, as prepared for TEM, were used for other tests.

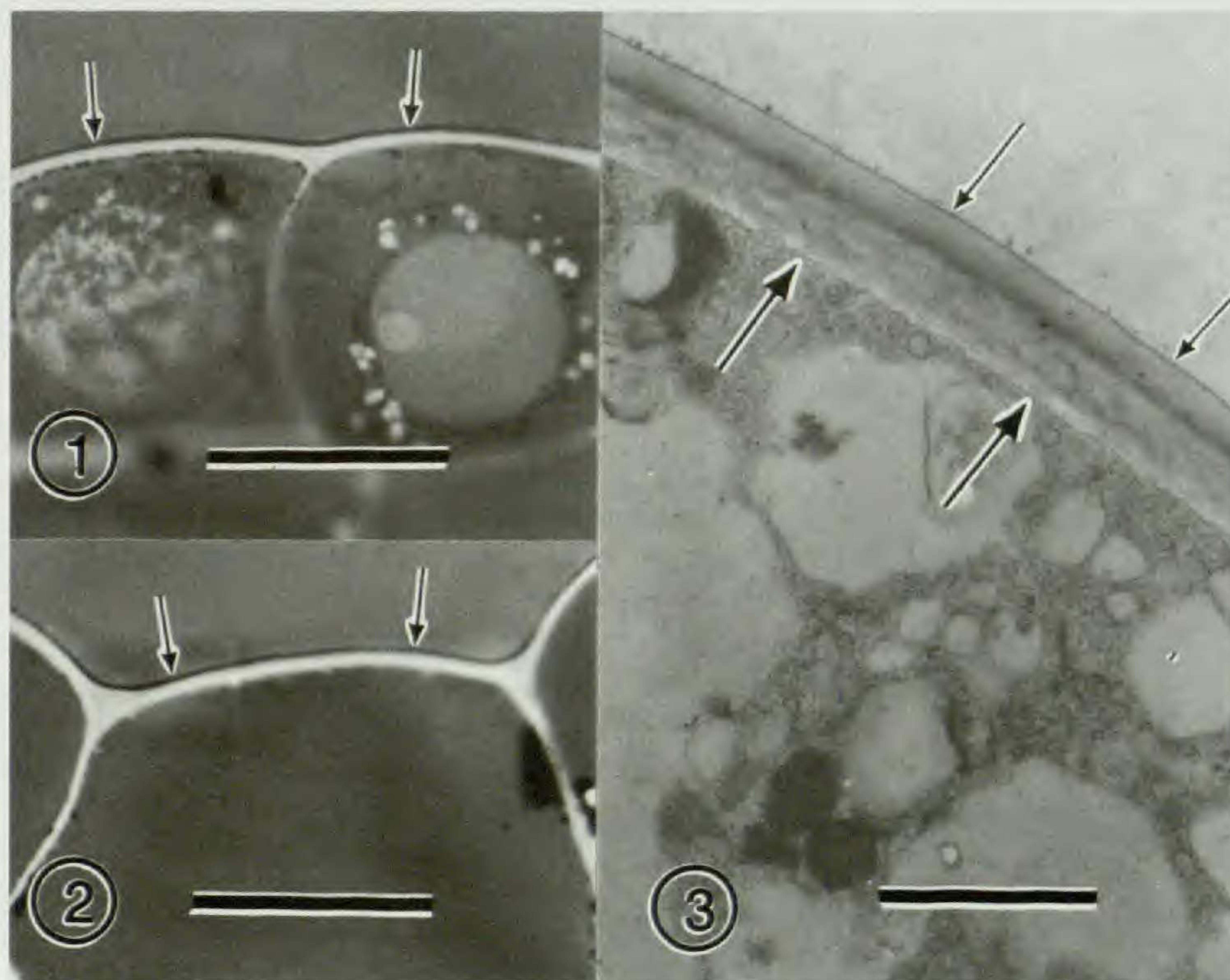
To determine the presence of lipids, hand sections were stained with Sudan IV (Jensen, 1962). Thick sections of gametophytes in Spurr's resin were stained for lipids with Sudan black B (Bronner, 1975). Lipids were extracted from the Spurr's material with the technique of Eurenius and Jarskar (1974) and from hand sections with a warm methanol-chloroform (1:1) mixture (Pearse, 1968). The IKI-sulfuric acid test (Jensen, 1962) was used to demonstrate cellulose and the ferric-ferricyanide test (Wilson and Peterson, 1983) was employed to show polyphenolic materials.

Resistance of a cell wall to concentrated sulfuric acid digestion is characteristic of walls containing cutin, suberin, or lignin (Wilson and Peterson, 1983). Gametophytes were digested with sulfuric acid for 18 hr. After this treatment, they were stained with Sudan IV for evidence of lipid. The phloroglucinal-HCl test (Jensen, 1962) was employed in an attempt to demonstrate lignin in these walls.

Results

The surface of *Tmesipteris* gametophytes has two layers (Figs. 1 and 2) and the outer layer is lipid material. This layer is observed in material embedded in Spurr's resin that has been sectioned and stained with Sudan black B (Figs. 1, 2). Hand sections of living gametophytes show the lipid layer stained with Sudan IV. In the preparations using the Sudan stains, the inner layer did not stain, although lipid droplets in the cytoplasm did (Fig. 1 and 2). The inner layer of the surface stains for cellulose and polyphenolics. Crushing the wall increased the cellulose staining with IKI-sulfuric acid. The outer lipid layer did not stain with the stains for nonlipid materials.

Distinct layers to the gametophyte surface can be seen with transmission electron microscopy (Fig. 3). Young gametophyte cells have two thick layers making up the surface (Fig. 3) and the outer lipid layer averaged about 30% of the surface thickness. The lipid layer is electron lucent and primarily amorphous and it abuts directly on the inner polysaccharide layer. There is a thin line of darker material at the outer surface of the polysaccharide layer that is not evident with light microscopy.



FIGS. 1–3. Light and electron microscopy of the surface of *Tmesipteris* gametophytes. **1.** Sudan Black B staining of the gametophyte surface (arrows) and small lipid droplets in the cells. The polysaccharide portion of the surface wall and starch grains in the cells are unstained and white. **2.** Sudan black B staining of the gametophyte surface (arrows) and the unstained portion of the surface wall. **3.** The two-layered gametophyte surface, as demonstrated with electron microscopy, having an outer electron-lucent layer (small arrows) and an inner polysaccharide layer (large arrows). Scale bars in Figs. 1–2 = 30 μm and Fig. 3 = 2 μm.

Efforts were made to dissolve the outer lipid layer on the gametophyte surface. The technique of Eurenius and Jarskar (1970) removed lipid droplets from the cells embedded in Spurr's resin, but the outer lipid layer remained and stained with Sudan black B. Warm methanol-chloroform removed lipid from the cells in hand sections, but not the lipid staining material from the gametophyte surface. Treatment of gametophytes with sulfuric acid overnight did not digest the outer wall of the gametophytes. They remained intact, although the cell contents were destroyed. The lipid layer could still be stained with Sudan IV. The failure of the surface walls to be digested is an indicator of cutin, suberin, or lignin. A negative phloroglucinol-HCl test demonstrated the absence of lignin.

DISCUSSION

The earlier studies of Holloway (1917) and Lawson (1917a) did not report lipid on the surface of *Tmesipteris* gametophytes. However, the techniques (Whittier and Peterson 1995), which confirmed the presence of a lipid coating on *Psilotum* gametophytes, proved useful in studying the surface of *Tmesipteris* gametophytes. Positive staining of the gametophyte surface with

Sudan black B and Sudan IV and observations with electron microscopy demonstrated a lipid coating on *Tmesipteris* gametophytes. This layer appears to be the same as the lipid layer of *Psilotum* gametophytes.

Resistance of the gametophyte surface to sulfuric acid (Wilson and Peterson, 1983) and failure of organic solvents to extract the fatty material (Holloway, 1982b; Kolattukudy *et al.*, 1981) is an indication that cutin or suberin is present. Whether the coating is composed of cutin or suberin is unknown at this time. Simple staining procedures cannot distinguish one from the other (Wilson and Peterson 1983). The elaborate chemical analyses necessary to identify them (Kolattukudy, 1984) are beyond the scope of this study.

The surface wall of *Tmesipteris* gametophytes does not have the appearance of a suberized wall. The lipid layer is on the outer surface and not in the wall next to the plasma membrane, as would be expected for a suberized wall. Also, if the lipid material is suberin, it should have stained with stains for phenolic materials (Scott and Peterson, 1979), which it did not.

The lipid layer of these gametophytes is more similar to a cuticle than to a suberized wall. Its position as a surface layer on the external wall is typical of a cuticle. The amorphous structure of this layer is similar to that identified as a type 6 cuticle by Holloway (1982a). The lipid layer on *Tmesipteris* gametophytes, although possibly thinner, is basically the same as that on *Psilotum* gametophytes. It should be considered a cuticle until proven otherwise by critical chemical analyses.

Finding a cuticle on the surface of *Tmesipteris* gametophytes indicates that a gametophytic cuticle is characteristic of the Psilotaceae because gametophytes of both genera are now known to have them. It is not surprising that *Tmesipteris* gametophytes have a cuticle because they grow under the same or similar conditions as the rhizome of *Tmesipteris* and the gametophytes of *Psilotum*, both with cuticles. Both the gametophytes and rhizomes of *Tmesipteris* grow slowly in soil or humus and are long-lived. A cuticle would help to prevent the gametophytes from drying out, if there was drying of the soil or humus. Besides resistance to drying, the cuticle could help to protect the gametophytes from various biotic factors in their habitat. The occurrence of a cuticle on these gametophytes under the relatively moist conditions of axenic culture indicates that this is a permanent feature of these gametophytes and not just a response to drier conditions.

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